

5 interestingly, both sequences contains a predicted transmembrane domain (residues 455-477 in SEQ ID No.4 and 430-452 in SEQ ID No. 6) near their C-termini which indicates that the protease is anchored in the membrane. This feature is not found in any other aspartyl protease except Hu-Asp1.

10 **Example 3. Molecular cloning of mouse Asp2 cDNA and genomic DNA.**
Cloning and characterization of murine Asp2 cDNA—The murine ortholog of Hu_Asp2 was cloned using a combination of cDNA library screening, PCR, and genomic cloning.

15 Approximately 500,000 independent clones from a mouse brain cDNA library were screened using a ³²P-labeled coding sequence probe prepared from Hu_Asp2. Replicate
20 positives were subjected to DNA sequence analysis and the longest cDNA contained the entire 3' untranslated region and 47 amino acids in the coding region. PCR amplification of the same mouse brain cDNA library with an antisense oligonucleotide primer specific for the 5'-most cDNA sequence determined above and a sense primer specific for the 5' region
25 of human Asp2 sequence followed by DNA sequence analysis gave an additional 980 bp of the coding sequence. The remainder of the 5' sequence of murine Asp-2 was derived from
30 genomic sequence (see below).

Isolation and sequence analysis of the murine Asp-2 gene—A murine EST sequence encoding a portion of the murine Asp2 cDNA was identified in the GenBank EST database
35 using the BLAST search tool and the Hu-Asp2 coding sequence as the query. Clone
40 g3160898 displayed 88% shared identity to the human sequence over 352 bp.

Oligonucleotide primer pairs specific for this region of murine Asp2 were then synthesized
45 and used to amplify regions of the murine gene. Murine genomic DNA, derived from strain 129/SvJ, was amplified in the PCR (25 cycles) using various primer sets specific for murine Asp2 and the products analyzed by agarose gel electrophoresis. The primer set Zoo-1 and
25 Zoo-4 amplified a 750 bp fragment that contained approximately 600 bp of intron sequence based on comparison to the known cDNA sequence. This primer set was then used to
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5 screen a murine BAC library by PCR, a single genomic clone was isolated and this cloned
was confirmed contain the murine Asp2 gene by DNA sequence analysis. Shotgun DNA
sequencing of this Asp2 genomic clone and comparison to the cDNA sequences of both
10 Hu_Asp2 and the partial murine cDNA sequences defined the full-length sequence of
murine Asp2 (SEQ ID No. 7). The predicted amino acid sequence of murine Asp2 (SEQ ID
No. 8) showed 96.4% shared identity (GCG BestFit algorithm) with 18/501 amino acid
15 residue substitutions compared to the human sequence (Figure 4).

Example 4: Tissue Distribution of Expression of Hu-Asp2 Transcripts:

Materials and Methods:

20 10 The tissue distribution of expression of Hu-Asp-2 was determined using multiple
tissue Northern blots obtained from Clontech (Palo Alto, CA). Incyte clone 2696295 in
the vector pINCY was digested to completion with *EcoRI/NotI* and the 1.8 kb cDNA insert
purified by preparative agarose gel electrophoresis. This fragment was radiolabeled to a
25 specific activity $> 1 \times 10^9$ dpm/ μ g by random priming in the presence of [α - 32 P-dATP]
(>3000 Ci/mmol, Amersham, Arlington Heights, IL) and Klenow fragment of DNA
polymerase I. Nylon filters containing denatured, size fractionated poly A⁺ RNAs isolated
30 from different human tissues were hybridized with 2×10^6 dpm/ml probe in ExpressHyb
buffer (Clontech, Palo Alto, CA) for 1 hour at 68 °C and washed as recommended by the
manufacture. Hybridization signals were visualized by autoradiography using BioMax XR
35 20 film (Kodak, Rochester, NY) with intensifying screens at -80 °C.

Results and Discussion:

Limited information on the tissue distribution of expression of Hu-Asp-2 transcripts
was obtained from database analysis due to the relatively small number of ESTs detected
40 using the methods described above (< 5). In an effort to gain further information on the
expression of the Hu-Asp2 gene, Northern analysis was employed to determine both the
25 size(s) and abundance of Hu-Asp2 transcripts. PolyA⁺ RNAs isolated from a series of
peripheral tissues and brain regions were displayed on a solid support following separation
under denaturing conditions and Hu-Asp2 transcripts were visualized by high stringency
45 hybridization to radiolabeled insert from clone 2696295. The 2696295 cDNA probe
visualized a constellation of transcripts that migrated with apparent sizes of 3.0kb, 4.4 kb
30 and 8.0 kb with the latter two transcript being the most abundant.

Across the tissues surveyed, Hu-Asp2 transcripts were most abundant in pancreas and brain with lower but detectable levels observed in all other tissues examined except thymus and PBLs. Given the relative abundance of Hu-Asp2 transcripts in brain, the regional expression in brain regions was also established. A similar constellation of transcript sizes were detected in all brain regions examined [cerebellum, cerebral cortex, occipital pole, frontal lobe, temporal lobe and putamen] with the highest abundance in the medulla and spinal cord.

Example 5: Northern Blot Detection of HuAsp-1 and HuAsp-2 Transcripts in Human Cell Lines:

A variety of human cell lines were tested for their ability to produce Hu-Asp1 and Asp2 mRNA. Human embryonic kidney (HEK-293) cells, African green monkey (Cos-7) cells, Chinese hamster ovary (CHO) cells, HELA cells, and the neuroblastoma cell line IMR-32 were all obtained from the ATCC. Cells were cultured in DME containing 10% FCS except CHO cells which were maintained in α -MEM/10% FCS at 37 °C in 5% CO₂ until they were near confluence. Washed monolayers of cells (3×10^7) were lysed on the dishes and poly A⁺ RNA extracted using the Qiagen Oligotex Direct mRNA kit. Samples containing 2 μ g of poly A⁺ RNA from each cell line were fractionated under denaturing conditions (glyoxal-treated), transferred to a solid nylon membrane support by capillary action, and transcripts visualized by hybridization with random-primed labeled (³²P) coding sequence probes derived from either Hu-Asp1 or Hu-Asp2. Radioactive signals were detected by exposure to X-ray film and by image analysis with a PhosphorImager.

The Hu-Asp1 cDNA probe visualized a similar constellation of transcripts (2.6 kb and 3.5 kb) that were previously detected in human tissues. The relative abundance determined by quantification of the radioactive signal was Cos-7 > HEK 292 = HELA > IMR32.

The Hu-Asp2 cDNA probe also visualized a similar constellation of transcripts compared to tissue (3.0 kb, 4.4 kb, and 8.0 kb) with the following relative abundance: HEK 293 > Cos 7 > IMR32 > HELA.

Example 6: Modification of APP to increase A β processing for in vitro screening

Human cell lines that process A β peptide from APP provide a means to screen in cellular assays for inhibitors of β - and γ -secretase. Production and release of A β peptide into the culture supernatant is monitored by an enzyme-linked immunosorbent assay (ELISA). Although expression of APP is widespread and both neural and non-neuronal cell lines